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LUMINESCENCE AND PECULIARITIES OF THE STRUCTURAL STATE OF CELL PROTEINS

[Following is the translation of an article by S. V. Konev, Ye. A. Chernitskiy, and Ye. I. Lin, Laboratory of Biophysics and Isotopes, AN BSSR, Minsk, published in the Russian-language periodical Biofizika (Biophysics) Vol XIII, No 6, 1968, pages 1040--46. It was submitted on 21 Aug 1967.]

On the basis of an investigation of the spectra of ultraviolet fluorescence of various tissues and organs of a frog an assumption is made concerning the very hydrophobic nature of the micro-environment around the majority of triptophane residues of cell proteins, which is explained by the absence in the cells of significant quantities of proteins whose surface contacts with the aqueous medium. Differences are revealed in the processes of denaturation of proteins with detergent (0.4% sodium dodecylsulfate; pH 3.0) in solution and in the cell, in the latter case resulting in the formation of a "superhydrophobic" conformation of protein. By means of spectral luminescence and ATP-ase activity measurements recordings were made of conformation transitions in myofibrillar proteins under conditions of the cell and in solution under the effect of various concentrations of urea. The structural organization of the cell protects myosin from the denaturing effect of its solutions, which is manifested in the complete preservation of ATP-ase activity of cell myosin in 1 M urea and in residual activity (~20%) in 8 M urea.

Ultraviolet luminescence of cells, detected by Brumberg et al. [1; 2] and Vladimirov [3], has been dealt with in a considerable number of works [1-15]. At present it can be considered as proven that the luminescence of cells and their organoids - nucleus, mitochondria, and ribosomes - in the ultraviolet range of the spectrum is conditioned by proteins, and in them, primarily, by residues of the aromatic amino acid - tryptophan [9, 13-15]. This circumstance considerably eases the interpretation of luminescence data for an evaluation of the peculiarities of structural states and conformation transformations of proteins directly in the living intact cell.

In previous works [13-15] in an example of various tissues and organs of a frog and certain microorganisms it was revealed that there was a considerably more short-wave position of tryptophan spectra of cell fluorescence in comparison with proteins in solutions, which was interpreted as intensification of the hydrophobic nature of the micro-environment of tryptophan residues of proteins,

incorporated in the composition of cell structures, and connected with the influence of quartenary and supermolecular structures.

In the present work results are cited from further investigations of the peculiarities of structural states of proteins in the cell and changes in them under the influence of denaturing factors - urea and detergent - by using the luminescence method.

Objects and Methods of Investigation

In the work we used various tissues of the frog Rana temporaria. These were isolated from an animal which had been rendered motionless by disruption of the spinal cord with as little damage as possible. To prevent drying during the measurements the tissues were kept in Ringer solution. In the work we used urea of an analytical grade brand and sodium dodecylsulfate ("Reanal", Hungary). The investigated tissue was placed in a rectangular quartz cuvette from an SF-4 spectrophotometer which contained Ringer solution and pressed slightly to the optical face of the cuvette.

Excitation and measurement of luminescence were carried out from the forward wall of the cuvette on a unit made from two quartz monochromators which was described previously [16]; sensitivity of the unit was controlled by an aqueous solution of tryptophan. Since during excitation by light with a wavelength $\lambda_{ex} = 280$ nm for a number of organs the tyrosine component of luminescence was observed, the spectra of fluorescence were recorded at $\lambda_{ex} = 296$ nm. The unit was graduated for spectral sensitivity with the help of a band tube.

Sarcoplasm proteins, actin, and myosin were removed from the gastrocnemius muscle of the frog by the method of extraction by saline solutions of various ionic strength [17]. ATP-ase activity of myosin was determined by mineral phosphorus, forming during the hydrolysis of ATP [17].

Results and Their Discussion

The table presents the position of maximums of tryptophan fluorescence of various tissues and organs of the frog. Certain differences are apparent in the position of maximums of spectra of fluorescence which are connected with the tissue affiliation of the cells.

Nevertheless, for cells of all the investigated tissues a more shortwave position of spectra of fluorescence is characteristic in comparison with the majority of proteins in solution [18] and also with sarcoplasm and myofibrillar proteins which were extracted from the muscles. Attention is drawn to the circumstance that the spectra of fluorescence of various tissues are close, though shifted slightly to the long-wave side, in comparison with the spectrum of fluorescence of chymotrypsinogen, containing residues of tryptophan

which are found exclusively within protein globules in a hydrophobic micro-environment [18, 19].

Positions of maximums of fluorescence of tissues of a frog and proteins under various conditions

(a) Объект исследования	(b) Положение максимума флуоресценции, мкм		
	(c) в растворе Рингера	в 0,4%-ном додецил-сульфате Na, pH 3 (d)	в 8 М мочевине (e)
(f) Мышца	331	330	337
(g) Мозг	333	330	339
(h) Язык	332	330	337
(i) Легкое	331	328	330
(j) Эпителий пищевода	334	332	337
(k) Печень	335	330	338
(l) Кожа	332	331	339
(n) Химотрипсиноген	331		—
(m) Саркоплазматические белки (мышцы в 0,03 *М* KCl)	336	340—341	349
(o) Миофибриллярные белки (в растворе Вебера)	336	341	340

Key: a-Object of investigation; (b) Position of maximum of fluorescence, nm; (c) in Ringer solution; (d) in C.4% Na dodecylsulfate, pH 3; (e) in 8 M urea; (f) Muscle; (g) Brain; (h) Tongue; (i) Lungs; (j) Epithelium from esophagus; (k) Liver; (l) Skin; (m) Sarcoplasm proteins (muscles in 0.03 M KCl); (o) Myofibrillar proteins (in Weber solution). [TN: (n) Chymotrypsinogen]

Therefore it is possible to think that the overwhelming majority of residues of tryptophan of cell proteins are also found in a sufficiently hydrophobic nonpolar micro-environment *, i.e., that the cells practically do not contain proteins whose surface is in contact with the aqueous medium ("bare protein"). This in its turn may be the result of the influence of quatenary and supermolecular structures of a cell, incorporation in the composition of which is accompanied by the screening of a large portion of the surface of macromolecules of protein by neighboring molecules (for example, other proteins or lipids).

* At present the generally accepted point of view is that the position of the maximum of fluorescence of tryptophan residues in protein is determined mainly by the polarity of the micro-environment [10, 12, 14, 18].

The influence of the supermolecular organization of a cell on the physicochemical state of its proteins is manifested also in the case of the action of high concentrations of denaturing substances - urea and Na dodecylsulfate - causing in proteins two effects correspondingly: 1 - disintegration of globules with the transfer of tryptophan residues into the polar micro-environment [18], and 2 - supplementary spiralization of protein with the development around the globules of a low-polarity membrane made from connected hydrocarbon fragments of molecules of detergent [20-22]. Just as in the case of proteins in solution, the maintenance of tissues in 8 M urea for one hour is accompanied by a long-wave shift of their spectra of fluorescence, which was noted in the previous work [15]. As a result of the influence of urea the maximums of spectra of fluorescence of cells of various origin vary somewhat in their position (λ_{max} = 337--339 nm), without achieving, however, that long-wave maximum which is characteristic for free proteins in solutions of 8 M urea (348--350 nm), and also those values which are characteristic for protein extracts of muscle in 8 M urea (349 nm).

The effect of 0.4% Na dodecylsulfate leads, on the contrary, to that short-wave shift as a result of which the maximums of spectra of fluorescence for a number of organs turn out to be disposed in even a more short-wave range than for the most "hydrophobic" protein - chymotrypsinogen (see Table) [TN. Note the reversing of M and n on the paste-up.]

It is characteristic that for protein extracts of muscle, and also for chymotrypsinogen a long-wave shift is observed here. It may be thought, therefore, that the intracellular organization alters in a significant manner the nature of the interaction of detergent with protein, as a result of which there are definite changes of structure within the globules, leading to a spatial redistribution of amino acid radicals and a lessening of the degree of polarity of the micro-environment not only around "external" but also "internal" residues of tryptophan. These structural reorganizations, in muscle proteins in particular, under the action of Na dodecylsulfate on them are accompanied by the complete suppression of ATP-ase activity of myosin.

For the purpose of a more detailed study of the role of structural factors and intermolecular interactions within the cell (supermolecular structures) in processes of intramolecular conformation reorganizations of protein which are taking place directly in the cell, we investigated the comparative action of various concentrations of denaturing agent (urea) on proteins of the actomyosin complex in solution and in the cell (muscle tissue).

In the course of the experiments, along with the spectra of fluorescence the ATP-ase activity of myosin was determined.

As can be seen from Fig. 1 and 2, outside of the cell, in solution, myosin under the influence of urea is capable of undergoing at least two different structural transitions. Monomolar solutions of urea already lead to a long-wave shift of spectra of fluorescence and a drop of ATP-ase activity by 50-60%. The transition bears an irreversible nature (restoration of fermentative activity and position of maximums of fluorescence is not observed following removal of urea by dialysis). Apparently a certain residual ATP-ase activity of myosin in 2--4 M urea (Fig. 1) is connected with the insufficient time of action of the denaturing agent, since after prolonged dialysis a solution of myosin in 2 M urea did not possess ATP-ase activity. Therefore it can be thought that the conformation transition, connected with losses of fermentative activity of myosin and the long-wave shift of its spectra of fluorescence by 2 nm, may be completely finished already in solutions of 2 M urea.

Further structural changes (second conformation transition or several transitions) are apparently caused by urea even in denatured biologically inactive protein. These post-denatured changes of protein are manifested in a progressive shift of spectra of fluorescence to the long-wave side (from 338 nm in 2 M urea to 349 nm in 8 M urea) and have a reversible nature: after dialysis solutions of myosin in 4, 6, and 8 M urea acquire the same spectra of fluorescence which are characteristic for solutions of myosin in 2 M urea with a maximum at 338 nm (Fig. 2). Thus the result of a joint denaturing--renaturing process during the influence of any high (4-8 M) concentrations of urea is the emergence of one and the same thermodynamically stable conformation of myosin, devoid of fermentative activity and possessing a spectrum of fluorescence with a maximum at 338 nm.

Losses of ATP-ase activity of myosin against a background of a comparatively insignificant long-wave shift of spectrum of fluorescence (by 2 nm) and the capacity of inactive myosin for a subsequent significant (by 11 nm) spectral shift following exposures to large concentrations of urea testify simultaneously that the process of inactivation is not accompanied by the coarse unfolding of globules of protein, and that only in the course of post-denatured structural reorganizations does that intensive disintegration of protein globule take place at which residues of tryptophan fall into an almost aqueous, hydrophilic micro-environment (the positions of maximums of fluorescence of an aqueous solution of tryptophan and myosin in 8 M of urea almost coincide).

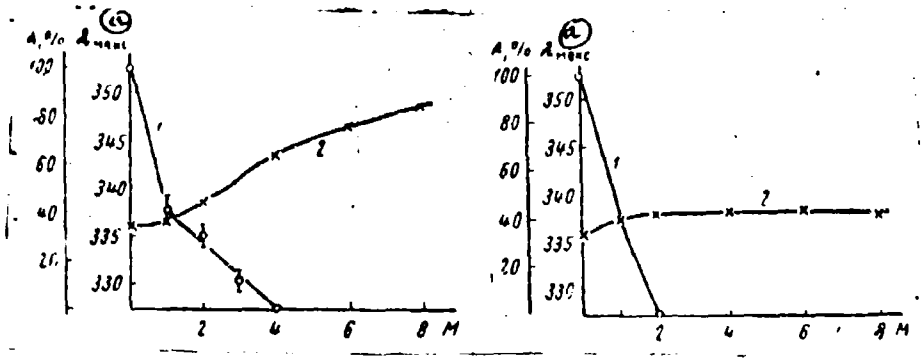

Fig. 1. Influence of various concentrations of urea on the ATP-ase activity (curve 1; ordinate A, %) and position of fluorescence of solutions of myosin (curve 2; ordinate λ_{max}, nm). Measurements made in the presence of urea.
Key: (a) λ_{max}.

Fig. 2. Influence of various concentrations of urea on the ATP-ase activity (1) and position of maximum of fluorescence of solutions of myosin (2). Measurements made after removal of urea by dialysis. Designation of ordinates explained in Fig. 1.
Key: (a) λ_{max}.

These conclusions agree well with data, based on a study by other methods, concerning conformation shifts taking place under the influence of urea on myosin. Small, Harrington, and Kielley [23] showed that in solutions of 2 M urea changes in viscosity do not occur, though a certain lessening is observed in the degree of α-spiralization of protein. Under the influence of 4--8 M urea an increase of viscosity is observed as well as the complete fusion of spirals. This additional change in the degree of spiralization of protein has a reversible nature, i.e., after removal of urea from such solutions myosin acquires the same values of optical rotation [a] which are characteristic for the conformation of protein in 2 M solutions of urea. Kasuya and Takashina [24] observed an increase in the average distances between residues of tryptophan and molecules of dye which were chemically fixed in the structure of protein under the influence of 1--2 M solutions of urea on myosin.

A considerably high degree of complexity and uniqueness distinguish the picture of interaction of myosin with various concentrations of urea in muscle tissue. First of all, in contrast to myosin in solution, myosin within the cell did not change its ATP-ase activity during prolonged (1-2 hours) exposure of muscle in a solution of 1 M urea. At the same time with the same influence, but after removal of urea by dialysis, a small and reversible shift (by 2 nm) is observed in the spectrum of fluorescence of whole muscle (Fig. 3).

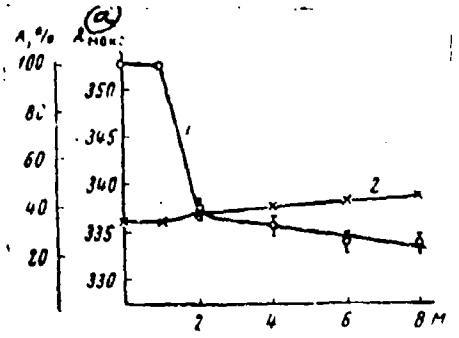

Fig. 3. Influence of various concentrations of urea on ATP-ase activity (1) and position of maximum of fluorescence (2) of myosin, found during the action of urea in muscle. Designations of ordinates the same as in Fig. 1.
Key: (a) λ_{max}.

Already this circumstance produced a slightly probable hypothesis that surface membranes of muscle tissues possess an impermeability in respect to monomolar solutions of urea. Moreover, special tests on measuring the amount of urea extracted by muscle tissue from solution showed that the cells are impermeable only for 0.5 M solutions of urea, while for monomolar solutions of urea its equilibrium concentration within the cells is even considerably higher (2.5 M) than outside of them and in the surrounding solution (1 M).

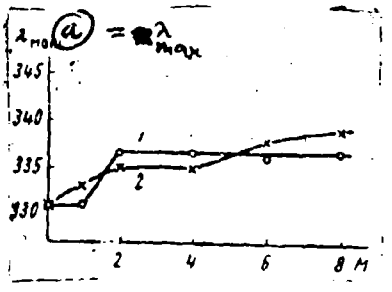

Fig. 4. Influence of various concentrations of urea on the position of maximums of fluorescence of muscle prior to dialysis (2) and after dialysis (1).

It can be assumed further that myosin within the muscle is protected from attack on the part of molecules of urea by some barrier, for example, sarcoplasm proteins, which actively combine with urea and thus create a protective effect with a simultaneous change of spectrum of fluorescence of muscle. Although such a possibility

cannot be completely excluded, nevertheless its realization in pure form is not very probable, if it is taken into consideration that: 1) according to the findings of Shtrankfel'd [25] the main contribution in luminescence of muscle is made primarily by contracting proteins of the actomyosin complex; 2)for whole muscle in monomolar urea an increase of intensity of fluorescence is observed [25] and a shift of its spectrum to the long-wave side (Fig. 4); 3) the main effect of sorption of urea by muscle proteins is observed namely for monomolar, and not more concentrated solutions of it.

In connection with this, more preferable is the following interpretation of effects of the action of monomolar solutions of urea on muscle. Under the conditions of intracellular structural organization of muscle the urea which penetrates into the cells attacks mainly the supermolecular (quaternary etc.) structures. Changes in the level are manifested in an increase in the intensity of fluorescence [25] and a long-wave shift of its spectra. At the same time intermolecular interactions (protein - protein, protein - lipoids, etc.) prevent the possibility of conversion of myosin from a native into denatured conformation, which is expressed in the inalterability of the level of its ATP-ase activity and spectra of fluorescence of contracting proteins, extracted from muscles which are found in a 1 M solution of urea (Fig. 3). It is characteristic that in solutions of monomolar urea in muscle there is observed only a temporary suppression of physiological excitability, replaced after a short period of time by its complete restoration [26]. The true intramacromolecular conformation transition in contracting proteins of muscle shifts, in comparison with proteins in solution, to the area of higher concentrations of urea.

As can be seen from Fig. 3, with a transition from 1 to 2 M solutions of urea there is a sharp drop in the level of ATP-ase activity of muscle myosin (by 60%) which is accompanied by a long-wave shift of spectra of fluorescence of proteins of the Weber fraction. In the same area there is observed an additional shift of spectrum of fluorescence of whole muscle. It has an absolute value which is close to that for the active--denatured protein transition in solution (Fig. 4).

It is necessary to accept, therefore, that with a transition from 1 to 2 M solutions of urea there is a conformation transition in contracting proteins of muscle which is accompanied by a drop in ATP-ase activity. It is namely in solutions of 2 M urea that muscle loses its excitability [26]. Attention is attracted to two characteristic peculiarities of this conformation transition which is taking place within the cell: 1) ATP-ase activity is not lost completely, as this is observed for solutions, but only partially (approximately by 80%); here the 20% residual activity is preserved right up to the exposure of muscle to 8 M solutions of urea;

2) removal of urea from muscle by means of equilibrium dialysis against physiological solution leads to a shift of spectra of fluorescence in the same long-wave direction as its introduction. In other words, both the introduction and the subsequent removal of urea causes effects which are similar in nature - an increase in the degree of hydrophilization of residues of tryptophan of muscle proteins, which is not noted for myosin in solution. For concentrations of urea in the external solution in an interval of 2--4 M a characteristic is the absence of a subsequent significant drop in ATP-ase activity, a shift in the spectrum of fluorescence of whole muscle, or the nature of spectral changes after dialysis (Fig. 5).

Therefore it can be thought that structural changes, additional to those which already took place in 2 M urea, do not take place in this interval of concentrations of urea.

However, under the influence of 6 and 8 M solutions of urea there is observed a further, but already reversible long-wave shift of spectra of whole muscle. Therefore it can be thought that under the influences of 6--8 M urea in muscle there are subsequent structural changes which do not affect the functional activity of myosin.

It is also apparent from Fig. 4 that regardless of what concentrations of urea are acting on the muscle (except 1 M urea) and to which structural changes they lead, the removal of urea is accompanied by the transition of muscle proteins always to the same structural state which is characteristic for the maximum of fluorescence of muscle at 337 nm.

This circumstance gives a certain foundation to the assumption that in the absence of urea contracting proteins of muscle can be found in two diverse thermodynamically stable conditions: 1) native, biologically active (form 331 nm), and 2) changed, partially fermentatively active condition (form 337 nm). The second condition emerges from the first after the influence of 2--8 M urea with its subsequent removal under conditions of the cell.

As already pointed out above, outside of the cell, in solutions of 4--8 M urea myosin is found only in a fermentatively inactive conformation, not capable of renaturation after removal of urea.

However, it is still unclear if the possibility of existence of a partially active form of myosin is connected with its heterogeneity in muscle or with the fact that the nature of conformation transitions or the process of denaturation of all the molecules of myosin within the muscle proceeds differently than in solution, leading to the formation of an end product, different in its conformation, but possessing fermentative activity. Apparently certain approaches to the solution of the problem may be given by the study of the kinetic characteristics of activity of myosin, isolated from denatured urea and renatured muscle (determination by the Michaelis constant).

Thus the peculiarity of the denaturation-renaturation processes, connected with the action of urea on myosin in a cell, in comparison with myosin in solution, can be reduced to three basic moments: 1) shift of concentration thresholds of conformation transitions; 2) preservation of partial ATP-ase activity of myosin at any concentrations of denaturing agent; 3) change in the nature of reversibility of post-denaturing structural reorganizations within muscle, which is manifested in a reconstruction of conformation, corresponding not to primary (2 M urea), but to secondary structural transition (5 M urea).

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